

4. Experiments corresponding to the description of the use of a delivery mixture comprising a delivery agent consisting of a dendrimer mixed with a nucleic acid capable of mediating RNA interference were carried out in my laboratory by Ya-Lin Chiu under my direction and supervision.

5. Exhibits 1-11 are copies of notebook entries from the laboratory notebook of Ya-Lin Chiu, titled "Delivery Method and Localization of siRNA," for the period October 10, 2002 through February 11, 2003. Exhibits 1-11 are notebook entries of experiments carried out during the period October 21, 2002 through November 19, 2002.

a. Exhibit 1 is an outline of experimental protocol used in experiments dated 10/21/02 – 10/25/02 for determination of transfection efficiency of siRNA by various delivery agents, including PAMAM dendrimer and lipofectamine.

b. Exhibit 2 is a description of the PAMAM dendrimer used in experiments. A PAMAM G4 dendrimer in a 90 µg/µl methanol solution was used.

c. Exhibit 3 is fluorescence level results of siRNA transfection experiments carried out 10/23/02.

d. Exhibit 4 is a computer printout of fluorescence results of transfection experiments using PAMAM G4 dendrimer and Lipofectamine, and carried out 10/23/02.

e. Exhibit 5 is graph results of transfection experiments introducing Cy3-labeled EGFP duplex siRNAs using PAMAM G4 dendrimer and Lipofectamine carried out 10/23/02. A note in the page indicates the product code number in the Aldrich catalog for the PAMAM Generation 4 dendrimer used in experiments. Exhibit 8 is a graph summary of results of similar transfection experiments introducing Cy3-labeled CDK9 duplex siRNAs carried out 10/23/02.

f. Exhibit 6 is protein levels of lysates from cells transfected with CDK9 siRNA by PAMAM dendrimer 10/29/02.

g. Exhibit 7 is immunoblot results of lysates from cells transfected with CDK9 siRNA by PAMAM dendrimer. Exhibit 9 is also immunoblot results of lysates from cells transfected with CDK9 siRNA by PAMAM dendrimer on 10/25/02.

h. Exhibit 10 and Exhibit 11 depict results of microscopic examination of HeLa cells transfected with Cy3-SS/AS siRNA by Lipofectamine or PAMAM, respectively on 11/19/02.

6. The notebook pages of Exhibit 2 and Exhibit 5 evidence use of generation 4 PAMAM dendrimer for transfection experiments. Each of the experiments carried out during the period 10/21/02 through 11/19/02 using a PAMAM dendrimer as a delivery agent used generation 4 PAMAM dendrimer.

7. Exhibits 1-11 are experiments and results corresponding to Example 1 (paragraph [0102] of the published application and FIGS. 1A and 1B), Example 2 (paragraph [0103] of the published application and FIG. 2), and Example 7 (paragraph [0111] of the published application and FIGS. 9A -9I) of the '176 application.

a. Example 1 and FIG. 1A and FIG. 1B correspond to the experiments and results depicted in Exhibits 1, 3, 4, 5, and 8.

b. Example 2 and FIG. 2 correspond to the experiments and results in Exhibits 6, 7, and 9.

c. Example 7 and FIG. 9 correspond to the experiments and results in Exhibit 11.

8. Exhibits 1-11 are experiments and results corresponding to Example 2 (page 19 line 23 to page 20, line 31 and FIGS. 1A and 1B), Example 3 (page 21, lines 1-21 and FIG. 2), and Example 8 (page 24, line 29 to page 25, line 14 and FIGS. 9A -9I) of U.S. provisional patent application no. 60/430,520.

a. Example 2 and FIG. 1A and FIG. 1B correspond to the experiments and results depicted in Exhibits 1, 3, 4, 5, and 8.

b. Example 3 and FIG. 2 correspond to the experiments and results in Exhibits 6, 7, and 9.

c. Example 8 and FIG. 9 correspond to the experiments and results in Exhibit 11.


9. The experiments described in the '176 application (Example 1 (paragraph [0102] of the published application and FIGS. 1A and 1B), Example 2 (paragraph [0103] of the published application and FIG. 2), and Example 7 (paragraph [0111] of the published application and FIGS. 9A –9I) of the '176 application) used a generation 4 PAMAM dendrimer as a delivery agent mixed with a nucleic acid capable of mediating RNA interference.

10. The experiments described in U.S. Provisional application 60/430,520 (Example 2 (page 19 line 23 to page 20, line 31 and FIGS. 1A and 1B), Example 3 (page 21, lines 1-21 and FIG. 2), and Example 8 (page 24, line 29 to page 25, line 14 and FIGS. 9A –9I) of U.S. provisional patent application no. 60/430,520) used a generation 4 PAMAM dendrimer as a delivery agent mixed with a nucleic acid capable of mediating RNA interference.

11. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Respectfully Submitted,

Date: 11/9/07

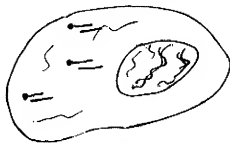

Tariq M. Rana

10/21/02 ~ 10/23/02 Determine transfection efficiency
Using cy3-labeled siRNA as indicator

EGFP 5'cy3-SS/AS Duplex

- ↓ Transfection by
1. Lipofectamine
 2. Nanoparticle #1
 3. PAMAM

Hela cell



on 60 mm plates

↓ 6 hr incubation at 37°C

↓ washed 3x by 5 mL PBS

↓ Qiagen DNA/RNA Extraction kit

↓ pellet
protein
Removed by strong denaturing reagent
followed by ϕ 13000rpm 20 min
4°C

↓ solution
containing DNA
RNA
small RNA

↓ Isopropanol
precipitation
4°C, 13000rpm 30 min

↓ Redissolve pellet in H₂O
(heat at 70°C 10 min)

↓ Detect cy3 signal
by Fluorometer

Yi-Lin Chen

EXHIBIT 1

PM AM-64

10/21/02

10g/110 mL.

90 ug/ul. in Methanol.

EXHIBIT 2

10/23/02

102302-1

102302-2

102302-4

102302-6

EGFP

RFP 2

EGFP

RFP

EGFP

RFP

18560

10690

18220

19400

11650

10570

8500

8867

9982

11080

10780

4126

5962

4698

2

272300

3

53180

4

252200

5

266600

6

286400

7

364800

8

587700

9

594500

10

194700

11

132900

12

89570

13

58490

14

54000

15

35040

16

26180

17

25970

18

28160

19

22080

104300

101900

98710

104300

119000

160300

276300

291400

87680

84660

86980

91380

91050

92920

106900

115800

135900

120500

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261400

218700

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208600

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	Cy3 Fluorescence Intensity	Treatment	Relative siRNA uptake Efficiency
1	18560.000	Lipofectamin (20ug)	1.000
2	10690.000	PAMAM(10ug)	0.576
3	18220.000	PAMAM(20ug)	0.982
4	19400.000	PAMAM(40ug)	1.045
5	11650.000	PAMAM(100ug)	0.628
6	10570.000	PAMAM(200ug)	0.570
7	8500.000	PAMAM(400ug)	0.458
8	8867.000	PAMAM(1mg)	0.478
9	7982.000	PAMAM 1 in PBS (10ug)	0.430
10	11080.000	PAMAM 1 in PBS (20ug)	0.597
11	10780.000	PAMAM 1 in PBS (40ug)	0.581
12	4126.000	PAMAM 1 in PBS (100ug)	0.222
13	5962.000	PAMAM 1 in PBS (200ug)	0.321
14	4698.000	PAMAM 1 in PBS (400ug)	0.253

EXHIBIT 4

Ja-Lin Chi

M

	OD750	Protein(ug/ul)	60ug	ul/60ug	Buffer
1	0.716	6.616	60.000	9.089	11.931
2	0.613	5.642	60.000	10.634	10.366
3	0.779	7.211	60.000	8.320	12.680
4	0.783	7.249	60.000	8.277	12.723
5	0.648	5.973	60.000	10.045	10.955
6	0.587	5.396	60.000	11.119	9.881
7	0.553	5.075	60.000	11.823	9.177
8	0.471	4.899	60.000	13.955	7.045
9	0.968	3.326	60.000	18.048	2.968

mock (40ug PAMAM only)
 100nM cdk9 siRNA by 20ug
 40ug
 100ug PAMAM-C
 200ug
 400ug
 1mg

EXHIBIT 6

m

Yan-Lin Chen

10/25/02 ~ 10/30/02

→ From 10/21/02 and 10/25/02 Experiment.

We know that PAMAM Dendrimer can send siRNA into the cell.
but cannot send the reporter plasmid in (size constraint?)
so ⇒ cannot use dual fluorescence assay to get Outtation data

→ Silencing of cdk9 expression by PAMAM mediated transfection

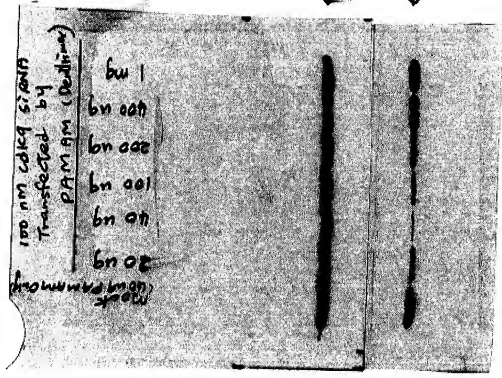
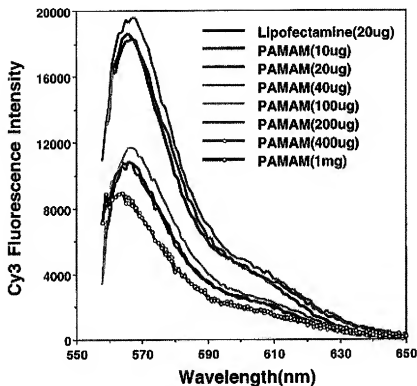


EXHIBIT 7

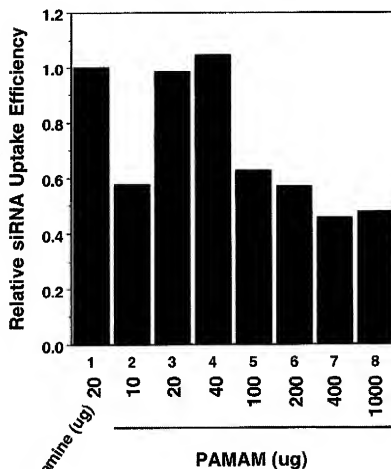
Fig 1

(A)



10/23/2002

(B)



Lipofectamine (ug)

PAMAM (ug)

EXHIBIT 8

m

Yu-Lin Chen

10/25/2002

Fig 2

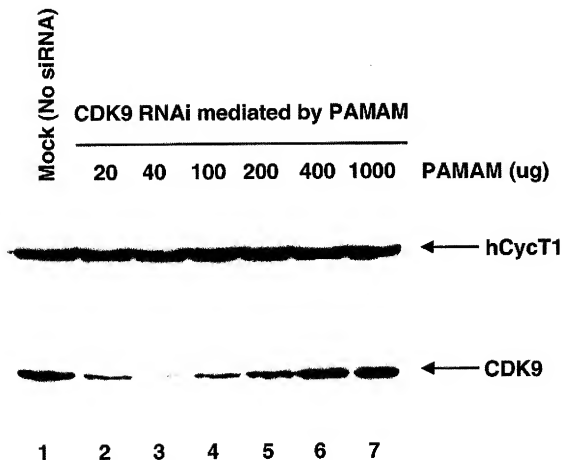
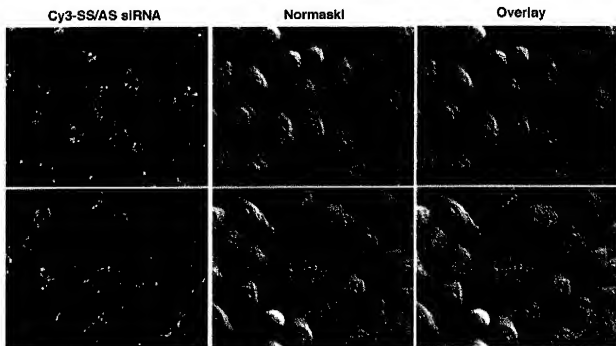


EXHIBIT 9

Ya-Li chin

11/19/02

Uptake of siRNA by HeLa Cells (20ug/1mL Lipofectamine-Mediated Transfection, 6h)(111902)



400X, 35 mm dish with coverslip bottom

Lelco Demo

EXHIBIT 10

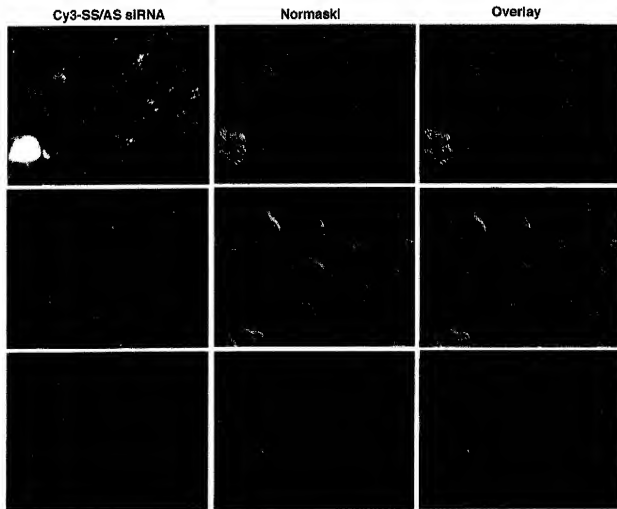
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11/19/2002

Ya-Lin Chiu

11/19/02

Uptake of siRNA by HeLa Cells (40ug/1mL PAMAM-Mediated Transfection, 6h)(111902)



400X, 35 mm dish with coverlip bottom

Leico Demo

EXHIBIT 11

TH

11/19/2002
✓ L-r